



(19)

Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 0 794 250 A1

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:
10.09.1997 Bulletin 1997/37

(51) Int. Cl.⁶: C12N 15/53, C12N 15/82,
C12N 9/02, C12N 5/10,
C12Q 1/68
// A01H5/00

(21) Application number: 97103098.6

(22) Date of filing: 26.02.1997

(84) Designated Contracting States:
BE ES FR GB IT NL

(30) Priority: 04.03.1996 CH 550/96

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(54) Isolation and sequencing of the hazel FAd2-N gene

(57) The invention relates to the isolation from hazel (*Corylus avellana L.*) of the FAD2-N gene coding for the $\Delta 12$ desaturase enzyme of the microsomal fraction and, in particular, provides the nucleotide sequence and the deduced amino-acid sequence of the gene and provides for its use as a probe for the isolation of other plant desaturases. It also relates to the use of this gene for altering the desaturase levels and consequently the fatty-acid composition of the plant.

Description

The present invention relates to the isolation from hazel (*Corylus avellana L.*) of the FAD2-N gene which codes for the $\Delta 12$ desaturase enzyme of the microsomal fraction.

More particularly, the invention relates to the nucleotide sequence, to the derived amino-acid sequence of the gene, and to its use as a probe for the isolation of other plant desaturases. It also relates to the use of this gene for altering the desaturase levels, and consequently the fatty-acid composition of the plant.

Alteration of the fatty-acid composition may have various applications in the industrial field. One of the greatest problems with hazelnuts is that they become rancid by oxidation. This is due to the auto-oxidation of unsaturated lipids with the consequent formation of volatile substances with a rancid odour which cannot easily be eliminated by the usual preservation systems. Amongst the possible strategies for reducing the tendency to become rancid, the best seems to be that of reducing the degree of unsaturation of the fatty acids present in the kernel oil, since susceptibility to auto-oxidation is positively correlated with this parameter. In fact, the rate of peroxide formation is correlated with the number of C=C double bonds in the fatty acids. The rate of auto-oxidation of the fatty acids in comparison with the oleate (18:1) is about 30 times greater in the linoleate (18:2) and 80 times greater in the linolenate (18:3). Moreover, the volatile substances resulting from the degradation of the linoleate and of the linolenate have a lower threshold of perception than those derived from the oleate. A reduction in linoleic acid should reduce the availability of substrates for lipoxygenase, reduce the loss of vitamin E during preservation, and reduce the production of volatile substances such as hexanal.

In the angiosperms, most of the synthesis of polyunsaturated lipids takes place by means of a single enzyme, that is, $\Delta 12$ (or $\omega 6$) desaturase (18:1 desaturase), of the endoplasmic reticulum, although there is an 18:1 chloroplast desaturase in the leaves of some plants. Moreover, this enzyme is responsible for more than 90% of the synthesis of polyunsaturated fatty acids in non-photosynthetic tissues such as, for example, in the kernels. The conversion of oleic acid (18:1) to linoleic acid (18:2) thus takes place by means of $\Delta 12$ desaturase, and from linoleic acid to linolenic acid (18:3) by means of $\Delta 15$ (or $\omega 3$) desaturase.

It has been shown with mutants of *Arabidopsis* that the FAD2 locus contains a gene which codes for the oleate desaturase enzyme of the endoplasmic reticulum (Okuley et al, 1994, *The Plant Cell* 6, 147-158). The FAD2 gene was in fact able to complement mutants of *Arabidopsis* which were deficient in desaturase activity of the endoplasmic reticulum. The gene coding for the same enzyme in soya has also recently been isolated and sequenced (Heppard et al, 1995, *Plant Physiol.*, in press).

A reduction in the $\Delta 12$ desaturase levels should therefore lead to a reduction in the linoleic acid content and, as a secondary effect, probably also to a reduction in linolenic acid. In hazelnuts the percentage of linoleic acid varies from 5 to 15%; the percentage of linolenic acid is from 0.1 to 0.2%. A reduction in these fatty acids should therefore be useful in the preservation of hazelnuts. There is therefore clearly a need to isolate the gene which codes for the $\Delta 12$ desaturase of the endoplasmic reticulum. The sequence of the gene could thus be used for gene inactivation in hazelnut kernels. This inactivation could be carried out either by the antisense technique (Smith et al. (1988) *Nature* 334, 724-726) or by the "transswitch" technique (Flavell (1994) *Proc. Natl. Acad. Sci. USA* 91, 3490-3496). In the antisense technique, the hazel would have to be transformed by the entire FAD2-N gene or by portions thereof, inserted in the opposite direction to the regulating sequences. In the "transswitch" technique, the hazel would have to be transformed by an identical copy of the FAD2-N gene.

The subjects of the present invention are defined by the following claims.

Embodiments of the present invention will now be described with reference to the following drawings, in which:

Figure 1 shows the restriction map of the N2 genome clone,

Figure 2 shows the nucleotide sequence of the hazel FAD2-N gene; the amino-acid sequence of the coding portion is also shown;

Figure 3 shows the nucleotide sequence of the "I" clone of cDNA,

Figure 4 shows a comparison between the nucleotide sequences of the "I" and "N2" clones,

Figure 5 shows a comparison between the amino-acids of the "N2" gene and $\Delta 12$ desaturases of *Arabidopsis* and of soya,

Figure 6 shows the homology between hazel $\Delta 12$ desaturase and various desaturases of other plants both plastid and of the endoplasmic reticulum,

Figure 7 shows the expression of the N2 gene in various varieties of hazel both in the leaves and in the kernels.

Isolation and cloning of the FAD2 gene of *Arabidopsis thaliana* for use as a probe

In order to isolate the gene which codes for hazel Δ 12 desaturase enzyme, it was necessary to use the FAD2 gene of *Arabidopsis* as a probe.

5 In order to isolate the *Arabidopsis* gene, two oligonucleotides were used as "primers" for the amplification of the sequences included between the start and the end of the gene. The oligonucleotides used were NOCC1 (CTGAATTCCAGGTGGAAGAATGCC) which contains the Eco RI restriction site and the sequences corresponding to the portion between bases 100 and 116 of the gene (Okuley J. et al, 1994, The Plant Cell 6, 147-158) and NOCC4 (AGGAATTCGACAATTCTTCACCATCATGC) which contains the restriction site of the Eco RI enzyme and the sequences complementary to the portion between base 1245 and base 1266. The amplification reaction was as follows: 12.8 μ l H₂O, 2.5 μ l 10 x PCR buffer (Perkin Elmer), 2.5 μ l *Arabidopsis* genome DNA(10 ng/l), 1 μ l dNTP, each 2.5mM, 2 μ l 25mM MgCl₂, 1 μ l NOCC1 oligonucleotide (50ng/ μ l), 1 μ l NOCC4 oligonucleotide (50ng/ μ l) 0.2 μ l Taq I DNA polymerase (Perkin Elmer) (5U/ μ l). The mixture thus prepared was subjected to 1 denaturing cycle for 1 minute at 94°C and to 40 cycles composed as follows: 30 seconds at 94°C, 1 minute at 52°C, 2 minutes at 72°C. The amplification products were separated on 1% agarose gel in TAE buffer (0.04M Tris-acetate, 0.002M EDTA) and stained with ethidium bromide at a concentration of 0.5 μ g/ml. The portion of gel containing the fragment of the expected length was withdrawn. In order to extract the DNA, 10 μ l of Qiaex resin (Qiaex extraction kit, firm Qiagen) were added for each 200mg of gel. The supplier's method was then followed. The DNA was then supplemented with a tenth of a volume of 10XH buffer (Boehringer) and 20 units of Eco RI enzyme (Boehringer). After incubation overnight at 37°C, the DNA was precipitated with 0.1 volumes of 5M NH₄OAc and one volume of isopropanol. After 10 minutes at ambient temperature, the DNA was centrifuged for 20 minutes at 14000 rpm and the precipitate was washed with 70% ethanol. The DNA was resuspended in 15 μ l of H₂O. The concentration was determined on gel by comparison with a known standard.

25 The amplified fragment was inserted in the pUC18 vector. A ligation mixture was prepared as follows: 1 μ l pUC18 plasmid DNA cut with Eco RI (20ng), 1.5 μ l fragment amplified with NOCC1 and 4 (25ng), 1 μ l 10X ligase buffer (Boehringer), 1 μ l T4 DNA ligase (1U/ μ l) (Boehringer), 4.5 μ l H₂O. The reaction mixture was incubated at 14°C for 12 hours.

30 In order to prepare competent cells, the method based on the compound hexamino-cobalt chloride was used (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 1.76-1.81). 10 μ l of the ligation mixture were added to each aliquot of competent cells, defrosted on ice. After the cells had been incubated on ice for 30 minutes they were subjected to thermal shock at 42°C for 90 seconds and were then replaced in ice for 60 seconds. After the addition of 0.5 ml of SOC broth (2% Bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 20mM glucose, pH7), the cells were incubated at 37°C with stirring for 90'. 100, 200 and 300 μ l aliquots were spread on plates containing solid LB broth (10gr/l NaCl, 10gr/l Bactotryptone, 5gr/l yeast extract, pH7.5, 15gr/l agar) with the addition of 50 μ g/ml of ampicillin and in the presence of IPTG and X-Gal. The plates were then incubated at 37°C overnight.

35 Some of the bacterial colonies obtained were first analyzed for their plasmid content by a quick method (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 1.32). The colonies containing a plasmid of the expected length were grown and their plasmid DNA extracted (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 1.33). Those containing a fragment of the expected length (1160 bp) were identified by digestion of the plasmid DNA with Eco RI. The E1 colony was selected.

40 One end of the insert of the E1 colony was sequenced. The plasmid DNA of the E1 clone was denatured and partially sequenced by Sanger's method using the enzyme Sequenase and ³⁵S-dATP (Amersham). The sequencing products were separated on 8% acrylamide, 8M urea, 1XTBE gel. After electrophoresis, the gel was dried and exposed overnight in contact with an autoradiographic plate (β max, Amersham). The sequence was compared with that published and was identical, identifying the *Arabidopsis* FAD2 gene in the cloned fragment.

45 Extraction of nucleic acids from hazel

50 Hazelnuts of the Nocchione, Montebello and San Giovanni varieties were harvested when almost fully ripe. The kernel was skinned before being used or frozen in liquid nitrogen. The leaves were harvested at a young stage and frozen in liquid nitrogen. 3 ml of extraction buffer were used for each gram of vegetable material with the use of the method described by Verwoerd et al. (Nucl. Ac. Res., 1989, 2362). Upon completion of the extraction, two selective precipitations were carried out by the addition of NaCl 2M, and 2 volumes of 95% ethanol to eliminate polysaccharides. The final pellet was resuspended in H₂O. Further centrifuging was then carried out to eliminate any non-resuspended material.

55 On the other hand, DNA was extracted from young leaves of the Nocchione and Montebello varieties. The vegetable tissue was pulverized in liquid nitrogen and the DNA extracted by the CTAB (REF) method. To eliminate the polysaccharides, NaCl 2M and 2 volumes of 95% ethanol were added. The samples were incubated for 15' at -80°C and centrifuged for 15' at 4°C and 14000 rpm (Eppendorf). This selective precipitation was repeated twice and the final pellet was resuspended in H₂O. Further centrifuging was then carried out to eliminate any non-resuspended material.

Checking of the probe on hazel DNA and RNA

About 20 μ g of DNA of the Montebello and Nocchione varieties was cut with Eco RI restriction enzyme in a volume of 300 μ l in the presence of 400 units of enzyme and H buffer (Promega), with incubation for one night at 37°C. After 5 digestion had been checked by gel electrophoresis of one twentieth of the reaction mixture, the samples were precipitated with ethanol and resuspended in 30 μ l of H₂O. The DNA was then subjected to electrophoresis on 0.7% agarose gel and transferred by capillarity onto nylon membrane (Southern blot) for one night in the presence of 20 x SSC (3M NaCl, 0.3M Na citrate). The membrane was dried in air for 30' and then fixed by UV treatment (120,000 μ J/cm²).

The *Arabidopsis* Δ 12 desaturase gene was used as a probe. For this purpose, the plasmid DNA of the E1 clone (5 μ g) was cut with 20 units of Eco RI in the presence of H buffer (Boehringer) in a volume of 30 μ l for 12 hours at 37°C. The insert of the clone was separated from the vector by electrophoresis on 1% agarose gel and extracted from the gel with the use of Qiaex resin in accordance with the suppliers' instructions (Qiagen). The DNA was denatured for 10' at 100°C, cooled rapidly in dry ice, and marked by the random priming method with the use of 6000 Ci/mmol (α^{32})P dATP and the reagents of Boehringer's marking kit.

15 The nylon membrane containing the hazel DNA was prehybridized for 1.5 hours at 55°C in standard buffer (5 x SSC, 0.1% (w/v) N-laurylsarcosine, 0.02% SDS, 1% blocking reagent solution) (10% blocking reagent solution: 10gr Boehringer blocking reagent in 150mM NaCl, 100mM maleic acid, pH7.5). The membrane was then hybridized with the *Arabidopsis* probe for one night at 55°C. The non-hybridized probe was washed twice for 15' in 2 x SSC, 0.1% SDS and twice for 15' each in 0.3 x SSC, 0.1% SDS, always at a temperature of 55°C. The probe remained coupled to the homologous sequences on the membrane was detected by autoradiography.

20 The RNA extracted from the young leaves of the Montebello and Nocchione varieties and from the kernels of the San Giovanni variety was separated on denaturing gel in the presence of formamide and transferred to nylon membrane by Northern blotting (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 7.43-7.45). 40 μ g/sample of total RNA extracted from San Giovanni kernels, Nocchione leaves and Montebello leaves were used. 25 60 pg of probe were used as a positive control. The RNA was loaded onto a 1% agarose gel in the presence of formaldehyde. The samples were then subjected to electrophoresis for 3 hours at 80 volts in the presence of 1xMOPS. The gel was rinsed in H₂O and then stained with ethidium bromide 0.5 μ g/ml to display the RNA. The RNA was then transferred onto a nylon membrane (Boehringer) by "capillary blotting" in the presence of 20 x SSC throughout the night at 4°C. After transfer, the membrane was dried on 3 MM paper and then fixed by crosslinking using UV light (Stratagene 30 UV Stratalinker 120000 μ J/cm²). The RNA was hybridised with the *Arabidopsis* Δ 12 desaturase probe as described for the DNA. Detection was carried out by autoradiography. The heterologous *Arabidopsis* probe was able to display a band with a molecular weight of about 1500 bp in the hazel RNA and 3 bands of about 18, 8 and 2.8 kb in the hazel DNA cut with Eco RI.

35 Construction of a gene library of cDNA

The gene library of cDNA was constructed from RNA from kernels harvested when almost fully ripe and taken from plants of the San Giovanni variety. For this purpose, the Poly(A)+mRNA was isolated from the total RNA with the use of the Poly(A)Tract mRNA Isolation System II, in accordance with the method provided by the firm Promega. The samples were eluted in H₂O and precipitated with 0.1 volumes of 3M NaOAc and 3 volumes of 95% ethanol. After one night at -80°C, the RNA was centrifuged for 15' at 14000 rpm (Eppendorf), the pellet was rinsed in 75% ethanol and resuspended in 10 μ l of H₂O. The concentration was read with a spectrophotometer and the yield was 3.2 μ g of Poly(A)+mRNA per mg of total RNA.

40 The messenger RNA polyadenilate derived from kernels of the San Giovanni variety was used as a template for the synthesis of complementary DNA (cDNA) with the use of Boehringer's "cDNA synthesis kit" in accordance with the method recommended by the suppliers. An extraction was then carried out with one volume of phenol:chloroform: isoamyl alcohol (25:24:1). The cDNA was then purified in a Pharmacia column (cDNA spun columns) after the addition of NaCl 100 mM. The buffer used was the following: 10mM Tris-HCl pH 7.5, 1mM EDTA, 150mM NaCl. Eco RI "adaptors" (Pharmacia) were added to the ends of the cDNA. The reaction mixture contained: 5 μ l of cDNA (half of the cDNA obtained from 6 μ g of Poly(A)+RNA), 10 μ l of ligase buffer 10 x (Promega), 10 μ l of Eco RI adaptors (0.01u/ μ l), 6 units of T4 DNA ligase (Promega), in a final volume of 100 μ l. After incubation for 12 hours at 12°C, the ligase enzyme was inactivated for 10' at 65°C. Phosphorylation of the adaptors then followed by the addition, to the 100 μ l mixture, of 10 μ l of 100mM ATP and 10 units of T4 polynucleotide kinase. After incubation at 37°C for 30', the enzyme was inactivated by incubation for 10' at 65°C. Purification was then carried out with one volume of phenol:chloroform:isoamyl alcohol (25:24:1). The cDNA was then purified from fragments of less than 400 bp as follows. After the addition of NaCl to a final concentration of 0.1M NaCl, the cDNA was separated by chromatography in a column with Sepharose CL-4B resin (Size prep 400 spun column, Pharmacia) according to the method suggested by the suppliers. The fragments of cDNA shorter than 400 bases were thus excluded. The cDNA was precipitated with one thirtieth of a volume of 3M NaOAc and 2 volumes of 95% ethanol, centrifuged and resuspended in 10 μ l of H₂O.

The cDNA was inserted in the λ phage vector Zap II cut with Eco RI and dephosphorylated (Stratagene) in the following manner: 2 μ l of cDNA (200 ng), 1 μ l of λ Zap II cut with Eco RI (1 μ g/ μ l) (Stratagene), 0.5 μ l of T4 DNA Ligase (4U/ μ l) (Promega), 0.5 μ l of 10 x ligation buffer (Promega), 1 μ l of H₂O. The reaction mixture was incubated for 14 hours at 12°C. The mixture containing the cDNA inserted in the vector was used for the reconstruction of the phages with the use of Stratagene's Gigapack Gold "in vitro packaging" kit. The gene library of phages thus obtained was constituted by about 300,000 pfu (plaque-forming units). In order to amplify the gene library, XL1 Blue MRF' cells were prepared as described by Stratagene and used the same day. The gene libraries were plated at a concentration of about 5000 pfu per plate (95 cm²). After growth, the phages were resuspended in SM (5.8gr/l NaCl, 2gr/l MgSO₄.7H₂O, 50ml/l 1M Tris HCl (pH 7.5), 5ml/l 2% gelatine) and, after the addition of chloroform to 5% and incubation for 15 minutes at ambient temperature, the cell debris was centrifuged for 10 minutes at 2000 x g. Chloroform to 0.3% was added to the supernatant liquid and the phages were preserved at 4°C. Aliquots were preserved at -70°C after the addition of DMSO to 7%. The gene library was titled.

Construction of a partial genome gene library

The DNA of the Nocchione variety was digested with Eco RI restriction enzyme and separated on agarose gel. The fragments with lengths of up to 10000 bp (base pairs) were isolated from the gel with the use of Qiaex resin according to the Qiagen's method. For cloning in the λ vector Zap II, 400ng of DNA fragments were incubated with 1 μ g of desphosphorylated λ Zap II (Stratagene) in the presence of ligase buffer and 1.5 units of T4 DNA ligase (Promega) for 12 hours at 14°C.

Stratagene's Gigapack Gold "in vitro packaging" kit was used in accordance with the suppliers' instructions to make up the gene library. The gene library of phages thus produced was amplified as described for the cDNA gene library. The complexity of the gene library was 1,500,000 clones. This gene library was also amplified.

Screening of the cDNA gene library

About 250,000 phages of the cDNA gene library were plated on LB broth in the presence of XL1 Blue MRF' cells, divided into 12 plates each containing 20,000 pfu. After growth, the phages were transferred onto nylon membranes and their denatured DNA was fixed on the membranes as described by Boehringer for screening with non-radioactive probes. The membranes were then hybridized with the *Arabidopsis* Δ 12 desaturase gene. The probe was prepared by the isolation of the insert containing the entire coding region of the gene from the plasmid. The insert was then marked with digoxigenin-dUTP with the use of Boehringer's "DNA labelling kit". Prehybridization was carried out in standard buffer (Boehringer) and hybridization was carried out in the same buffer with the addition of the *Arabidopsis* probe at a concentration of 10ng/ml and at a temperature of 55°C.

After washing twice in 2xSSC, 0.1% SDS for 5 minutes at ambient temperature and washing twice in 0.3xSSC, 0.1%SDS at 55°C, detection was carried out with the use of an anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer) and a chemiluminescent substrate (AMPPD, Boehringer).

11 positive phage plaques were identified. These were isolated, the phages resuspended in SM and titled. From 50 to 200 phages were plated for each positive plaque. The plaques were transferred onto nylon membranes and subjected to a second hybridization with the *Arabidopsis* Δ 12 desaturase probe, as already described above. The following clones which could hybridize with the *Arabidopsis* Δ 12 desaturase gene were obtained from the second screening: I, F, 4.

Screening of the genome gene library

The gene library of Nocchione DNA was subjected to screening in the same way as the cDNA gene library. 1,600,000 phages were plated, divided into 40 plates. After growth, they were transferred to nylon membranes as described for the cDNA gene library. The membranes were then hybridized with the *Arabidopsis* Δ 12 desaturase gene as described for the cDNA gene library. Autoradiography of the membranes showed 9 positive plaques. These plaques were isolated, titled and subjected to a second screening. 6 plaques were re-confirmed as positive. 4 of these gave a very strong signal.

Analysis of the clones isolated

The following positive phage clones were converted into plasmids by *in vivo* excision in accordance with the method suggested by Stratagene (Gigapack Gold in vitro packaging) : I, F, 4 (cDNA gene library), N2, N11, N17, N18, N21, N25 (genome gene library).

The plasmid DNA of the clones of the cDNA gene library was isolated and the length of the insert analyzed by digestion with Eco RI. The plasmid DNA of the genome clones was isolated, the length of the insert analyzed by cutting

with restriction enzyme, and the clones rechecked by hybridization with the *Arabidopsis* probe. Figure 1 shows the map of the N2 genome clone.

Sequencing

5 The N2 clone was selected from the genome clones. For sequencing, the insert was fragmented with Sau3A restriction enzyme and the fragments obtained were subcloned in pUC18 vector cut with BamHI (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 1.68-1.69). The clones obtained were analyzed both for the length of the insert and by hybridization with the *Arabidopsis* probe. Since the N2 insert was 2.8 kb and hence longer than the
 10 $\Delta 12$ desaturase gene, the hybridization excluded the clones containing sequences outside the gene. The insert of the I, F, 4 and N2 clones was isolated and sequenced with the use of the Sequenase kit and (35S)dATP. All of the clones (cDNA and genome) were first sequenced at the ends with the use of primers which could couple with the vector in both orientations. In order to complete the internal regions and to assemble the fragments of the N2 genome clone, internal
 15 oligonucleotides were then designed and synthesized and were used for the sequencing. The following table shows the sequences of the internal oligonucleotides:

OLIGONUCLEOTIDE	SEQUENCE
N2-3SS	CAG ACC AGC ATC CGA GAC
N2-3SD	GGA TTG GCT TAG GGG GGC
N2-29R'S	GCC AAC CAT GTC ATC AAC CC
NOCCS	ATG GTA GAG AAG AGA TGG TG
COL	CTG GTG GGT TGT TGA AG
N2-S1N	GGA GAG GTC ATA AAC AAC

30 The I and F clones were sequenced entirely. As far as the N2 clone is concerned, only the regions corresponding to the gene were sequenced. Figures 2 and 3 show their sequence. The I and F cDNA clones were identical. A comparison between I and the N2 genome clone showed the same sequence (Fig. 4), indicating that N2 contains the gene which codes for the cDNA of the I clone.

Comparison between the gene isolated and other desaturases

35 The nucleotide and amino-acid sequence of the N2 clone was compared with other desaturases (Figure 6). The greatest homology was with the two $\Delta 12$ desaturases of the endoplasmic reticulum and with a hydroxylase of ricin
 40 which uses the same substrate as $\Delta 12$ desaturase. Homology with the plastid $\Delta 12$ desaturases and with both the plastid and endoplasmic reticulum $\Delta 15$ desaturases was, however, much lower. Figure 5 shows the comparison between the amino-acid sequence of hazel $\Delta 12$ and those of *Arabidopsis* and soya.

Checking of the expression of the hazel $\Delta 12$ desaturase gene

45 RNA was extracted from kernels of the San Giovanni, Montebello and Nocchione varieties and from leaves of the Montebello and Nocchione varieties. After separation on agarose gel, the RNA was transferred onto a nylon membrane and hybridized with the insert of the I clone marked with digoxigenin. The result is shown in Figure 7, in which a band is visible in the kernel RNA but not in that of the leaves.

50

55

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(F) POSTAL CODE (ZIP): 6700

(ii) TITLE OF INVENTION: Isolation and sequencing of the
hazel FAD2-N
gene

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
(EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: CH 0550/96
(B) FILING DATE: 04-MAR-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1662 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Corylus avellana cv. Nocchione
(F) TISSUE TYPE: leaves

(vii) IMMEDIATE SOURCE:
(B) CLONE: N2

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 222..1370
(D) OTHER INFORMATION: /product= "delta-12 desaturase"
/gene= "Fad2"

EP 0 794 250 A1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

5 CCTCATAAAA AAGTAAGCTC ATTTACCTCA AGTAGGGTTT CCTTATGACA AATGAGTCCC
60

120 GCAATCCTTT TCTATGAGGT GCTATAATTG CAAATGTCCA AATCATAGGG ATATGGATCC

10 AAATACTATT AATATTATGT AGTGTGTTT TTTTTTCCC TCAAATTTAC TCTCACACCT
180

15 AAGTTGATTT TCTCCAGCAT TGGACATAGC CTCTGTAGAC A ATG GGA GCT AGA
233

Met Gly Ala Arg
1

281 AGC CGA ATG CCT GCT ACC AAC AAG CCT AAA GAG CAA AAA ACA CCC ATC

Ser Arg Met Pro Ala Thr Asn Lys Pro Lys Glu Gln Lys Thr Pro Ile

20 5 10 15 20

329 CAG CGA GCA CCA CAC ACA AAA CCC CCA TTC ACT CTT AGC CAA CTC AAG

Gln Arg Ala Pro His Thr Lys Pro Pro Phe Thr Leu Ser Gln Leu Lys

25 25 30 35

377 AAA GCC GTC CCA CCC AAT TGT TTC CAA CGC TCT CTC CTA CGC TCG TTC

Lys Ala Val Pro Pro Asn Cys Phe Gln Arg Ser Leu Leu Arg Ser Phe

40 45 50

425 TCA TAT GTT GTT TAT GAC CTC TCC TTA GCC TTC CTC TTC TAC TAT ATT

Ser Tyr Val Val Tyr Asp Leu Ser Leu Ala Phe Leu Phe Tyr Tyr Ile

45 55 60 65

473 GCT ACC TCT TAC TTC CAT CTC CTC CCT CAC CCC CTT TCC TAC TTG GCA

Ala Thr Ser Tyr Phe His Leu Leu Pro His Pro Leu Ser Tyr Leu Ala

45 70 75 80

521 TGG TCA ATC TAT TGG GCT CTC CAA GGC TGC ATT CTC ACC GGC GTT TGG

Trp Ser Ile Tyr Trp Ala Leu Gln Gly Cys Ile Leu Thr Gly Val Trp

50 85 90 95 100

55

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GTC ATC GCA CAT GAG TGC GGT CAC CAT GCC TTT AGT GAC TAC CAA TGG
569
Val Ile Ala His Glu Cys Gly His His Ala Phe Ser Asp Tyr Gln Trp
5 105 110 115

GTT GAT GAC ATG GTT GGC CTA ACC CTT CAC TCT GCT CTT TTA GTT CCA
617
Val Asp Asp Met Val Gly Leu Thr Leu His Ser Ala Leu Leu Val Pro
10 120 125 130

TAC TTT TCA TGG AAG ATT AGC CAC TGT CGC CAC CAC TCT AAC ACC GGC
665
Tyr Phe Ser Trp Lys Ile Ser His Cys Arg His His Ser Asn Thr Gly
15 135 140 145

TCC CTT GAC CGA GAT GAG GTG TTT GTC CCC AAG CCG AAA TCC AAA ATG
713
Ser Leu Asp Arg Asp Glu Val Phe Val Pro Lys Pro Lys Ser Lys Met
20 150 155 160

CCA TGG TTT TCT AAG TAC TTC AAC AAC CCA CCA GGT AGG GTC CTC ACT
761
Pro Trp Phe Ser Lys Tyr Phe Asn Asn Pro Pro Gly Arg Val Leu Thr
25 165 170 175 180

CTT TTG ATC ACA CTC ACT CTA GGC TGG CCC TTG TAC TTA GCC TTG AAT
809
Leu Leu Ile Thr Leu Thr Leu Gly Trp Pro Leu Tyr Leu Ala Leu Asn
30 185 190 195

GTT TCT GGC CGA CCC TAT GAT CGT TTT GCT TGC CAC TAT GAT CCC TAT
857
Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala Cys His Tyr Asp Pro Tyr
35 200 205 210

GGC CCC ATT TAT TCC AAT CGC GAA AGG TGT CAA ATA TTT GTC TCG GAT
905
Gly Pro Ile Tyr Ser Asn Arg Glu Arg Cys Gln Ile Phe Val Ser Asp
40 215 220 225

GCT GGT GTC TTT GCT ACA ACT TAT GTG CTT TAC TAC GCA GCA ATG TCA
953

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Ala Gly Val Phe Ala Thr Thr Tyr Val Leu Tyr Tyr Ala Ala Met Ser

230

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AAA GGG CTG GCA TGG CTT GTA TTC ATT TAT GGT ATG CCA TTG CTC ATA
1001

Lys Gly Leu Ala Trp Leu Val Phe Ile Tyr Gly Met Pro Leu Leu Ile

10

245

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GTG AAT GGC TTC CTT GTA TTA ATC ACC TAC TTG CAG CAC ACT CAC CCT
1049

Val Asn Gly Phe Leu Val Leu Ile Thr Tyr Leu Gln His Thr His Pro

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20

GCA TTG CCG CAC TAT GAC TCA TCA GAA TGG GAT TGG CTT AGG GGG GCA
1097

Ala Leu Pro His Tyr Asp Ser Ser Glu Trp Asp Trp Leu Arg Gly Ala

280

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25

TTG GCG ACG GCG GAT AGA GAT TAC GGA ATG CTG AAT AAG GTT TTC CAC
1145

Leu Ala Thr Ala Asp Arg Asp Tyr Gly Met Leu Asn Lys Val Phe His

295

300

305

30

AAT ATC ATA GAC ACC CAT GTG GCT CAC CAT CTC TTC TCT ACC ATG CCT
1193

Asn Ile Ile Asp Thr His Val Ala His His Leu Phe Ser Thr Met Pro

35

310

315

320

CAT TAC CAT GCA ATG GAA GCC ACC AAA GCA ATC AAG TCA ATA TTG GGC
1241

40

His Tyr His Ala Met Glu Ala Thr Lys Ala Ile Lys Ser Ile Leu Gly

325

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45

AAA TAC TAC CAG TTT GAT GGC ACT CCA GTT TAC AAG GCA GTG TGG AGG
1289

Lys Tyr Tyr Gln Phe Asp Gly Thr Pro Val Tyr Lys Ala Val Trp Arg

345

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355

50

GAG GCT AAA GAG TGC CTT TAT GTT GAG TCG GAC GAG GGG GCC CCT AAC
1337

Glu Ala Lys Glu Cys Leu Tyr Val Glu Ser Asp Glu Gly Ala Pro Asn

55

360

365

370

5 AAA GGT GTT TTC TGG TAT CAG AGC AAG CTG TGA TATTGGCTGG ATAGAGCCAA
 1390
 Lys Gly Val Phe Trp Tyr Gln Ser Lys Leu *

375

380

10 AGAAAATGTG ATTAGTAAGG TAGTGTCTTT GGTCAGTTG GTGTGTTAAG GAACAAATAA
 1450

15 TAATAATTAG CGACTATGAA TAGTTATTGT TAAACAAAAT TCACCCTTAT GTTTAGCAGG
 1510

AACTTTCTG GCTACACTT TTTTCGTATG AAAAGCGCAT ATTTTTAAAT TGTTATATTG
 1570

20 TTTGACATT ACTCAAGCTT CAAAATTAAT ATCACAGAAA ATATCCAATG TCGAAGGTTT
 1630

CATTGTAGGT TGAAAACTTT ATATTGAGGT GG
 1662

25 (2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 383 amino acids
 (B) TYPE: amino acid
 (C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

35 Met Gly Ala Arg Ser Arg Met Pro Ala Thr Asn Lys Pro Lys Glu Gln
 1 5 10 15

40 Lys Thr Pro Ile Gln Arg Ala Pro His Thr Lys Pro Pro Phe Thr Leu
 20 25 30

Ser Gln Leu Lys Lys Ala Val Pro Pro Asn Cys Phe Gln Arg Ser Leu
 35 40 45

45 Leu Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Ser Leu Ala Phe Leu
 50 55 60

50 Phe Tyr Tyr Ile Ala Thr Ser Tyr Phe His Leu Leu Pro His Pro Leu
 65 70 75 80

Ser Tyr Leu Ala Trp Ser Ile Tyr Trp Ala Leu Gln Gly Cys Ile Leu
 85 90 95

55

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Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His His Ala Phe Ser
100 105 110

5 Asp Tyr Gln Trp Val Asp Asp Met Val Gly Leu Thr Leu His Ser Ala
115 120 125

10 Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser His Cys Arg His His
130 135 140

Ser Asn Thr Gly Ser Leu Asp Arg Asp Glu Val Phe Val Pro Lys Pro
145 150 155 160

15 Lys Ser Lys Met Pro Trp Phe Ser Lys Tyr Phe Asn Asn Pro Pro Gly
165 170 175

20 Arg Val Leu Thr Leu Leu Ile Thr Leu Thr Leu Gly Trp Pro Leu Tyr
180 185 190

Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala Cys His
195 200 205

25 Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asn Arg Glu Arg Cys Gln Ile
210 215 220

30 Phe Val Ser Asp Ala Gly Val Phe Ala Thr Thr Tyr Val Leu Tyr Tyr
225 230 235 240

Ala Ala Met Ser Lys Gly Leu Ala Trp Leu Val Phe Ile Tyr Gly Met
245 250 255

35 Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile Thr Tyr Leu Gln
260 265 270

40 His Thr His Pro Ala Leu Pro His Tyr Asp Ser Ser Glu Trp Asp Trp
275 280 285

Leu Arg Gly Ala Leu Ala Thr Ala Asp Arg Asp Tyr Gly Met Leu Asn
290 295 300

45 Lys Val Phe His Asn Ile Ile Asp Thr His Val Ala His His Leu Phe
305 310 315 320

50 Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala Ile Lys
325 330 335

Ser Ile Leu Gly Lys Tyr Tyr Gln Phe Asp Gly Thr Pro Val Tyr Lys

340

345

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5 Ala Val Trp Arg Glu Ala Lys Glu Cys Leu Tyr Val Glu Ser Asp Glu
 355 360 365

Gly Ala Pro Asn Lys Gly Val Phe Trp Tyr Gln Ser Lys Leu *
 370 375 380

10 (2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
 15 (A) LENGTH: 1133 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

20 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: C-terminal

25 (vi) ORIGINAL SOURCE:

(A) ORGANISM: Corylus avellana L. cv. San Giovanni
 (D) DEVELOPMENTAL STAGE: Seed, storage deposition stage

(vii) IMMEDIATE SOURCE:

(B) CLONE: I

30 (ix) FEATURE:

(A) NAME/KEY: mRNA
 (B) LOCATION:1..1133
 (D) OTHER INFORMATION:/partial
 35 /gene= "Fad2"

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION:1..1019
 (D) OTHER INFORMATION:/partial
 40 /codon_start= 3
 /product= "delta-12 desaturase"
 /gene= "Fad2"

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TC CAA CGC TCT CTC CTA CGC TCG TTC TCA TAT GTT GTT TAT GAC CTC
 47 Gln Arg Ser Leu Leu Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu

50 385

390

395

TCC TTA GCC TTC CTC TTC TAC TAT ATT GCT ACC TCT TAC TTC CAT CTC

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95
Ser Leu Ala Phe Leu Phe Tyr Tyr Ile Ala Thr Ser Tyr Phe His Leu
5 400 405 410

CTC CCT CAC CCC CTT TCC TAC TTG GCA TGG TCA ATC TAT TGG GCT CTC
143
Leu Pro His Pro Leu Ser Tyr Leu Ala Trp Ser Ile Tyr Trp Ala Leu
10 415 420 425 430

CAA GGC TGC ATT CTC ACC GGC GTT TGG GTC ATC GCA CAT GAG TGC GGT
191
Gln Gly Cys Ile Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly
15 435 440 445

CAC CAT GCC TTT AGT GAC TAC CAA TGG GTT GAT GAC ATG GTT GGC CTA
239
His His Ala Phe Ser Asp Tyr Gln Trp Val Asp Asp Met Val Gly Leu
20 450 455 460

ACC CTT CAC TCT GCT CTT TTA GTT CCA TAC TTT TCA TGG AAG ATT AGC
287
Thr Leu His Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser
25 465 470 475

CAC TGT CGC CAC CAC TCT AAC ACC GGC TCC CTT GAC CGA GAT GAG GTG
335
His Cys Arg His His Ser Asn Thr Gly Ser Leu Asp Arg Asp Glu Val
30 480 485 490

TTT GTC CCC AAG CCG AAA TCC AAA ATG CCA TGG TTT TCT AAG TAC TTC
383
Phe Val Pro Lys Pro Lys Ser Lys Met Pro Trp Phe Ser Lys Tyr Phe
40 495 500 505 510

AAC AAC CCA CCA GGT AGG GTC CTC ACT CTT TTG ATC ACA CTC ACT CTA
431
Asn Asn Pro Pro Gly Arg Val Leu Thr Leu Leu Ile Thr Leu Thr Leu
45 515 520 525

GGC TGG CCC TTG TAC TTA GCC TTG AAT GTT TCT GGC CGA CCC TAT GAT
479
Gly Trp Pro Leu Tyr Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp
50

55

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	530	535	540
5	CGT TTT GCT TGC CAC TAT GAT CCC TAT GGC CCC ATT TAT TCC AAT CGC 527 Arg Phe Ala Cys His Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asn Arg		
	545	550	555
10	GAA AGG TGT CAA ATA TTT GTC TCG GAT GCT GGT GTC TTT GCT ACA ACT 575 Glu Arg Cys Gln Ile Phe Val Ser Asp Ala Gly Val Phe Ala Thr Thr		
15	560	565	570
	TAT GTG CTT TAC TAC GCA GCA ATG TCA AAA GGG CTG GCA TGG CTT GTA 623 Tyr Val Leu Tyr Tyr Ala Ala Met Ser Lys Gly Leu Ala Trp Leu Val		
20	575	580	585
	TTC ATT TAT GGT ATG CCA TTG CTC ATA GTG AAT GGC TTC CTT GTA TTA 671 Phe Ile Tyr Gly Met Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu		
	595	600	605
25	ATC ACC TAC TTG CAG CAC ACT CAC CCT GCA TTG CCG CAC TAT GAC TCA 719 Ile Thr Tyr Leu Gln His Thr His Pro Ala Leu Pro His Tyr Asp Ser		
	610	615	620
30	TCA GAA TGG GAT TGG CTT AGG GGG GCA TTG GCG ACG GCG GAT AGA GAT 767 Ser Glu Trp Asp Trp Leu Arg Gly Ala Leu Ala Thr Ala Asp Arg Asp		
	625	630	635
35	TAC GGA ATG CTG AAT AAG GTT TTC CAC AAT ATC ATA GAC ACC CAT GTG 815 Tyr Gly Met Leu Asn Lys Val Phe His Asn Ile Ile Asp Thr His Val		
40	640	645	650
	GCT CAC CAT CTC TTC TCT ACC ATG CCT CAT TAC CAT GCA ATG GAA GCC 863 Ala His His Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala		
45	655	660	665
50	670		

5 ACC AAA GCA ATC AAG TCA ATA TTG GGC AAA TAC TAC CAG TTT GAT GGC
 911 Thr Lys Ala Ile Lys Ser Ile Leu Gly Lys Tyr Tyr Gln Phe Asp Gly

675 680 685

10 ACT CCA GTT TAC AAG GCA GTG TGG AGG GAG GCT AAA GAG TGC CTT TAT
 959 Thr Pro Val Tyr Lys Ala Val Trp Arg Glu Ala Lys Glu Cys Leu Tyr

690 695 700

15 GTT GAG TCG GAC GAG GGG GCC CCT AAC AAA GGT GTT TTC TGG TAT CAG
 1007 Val Glu Ser Asp Glu Gly Ala Pro Asn Lys Gly Val Phe Trp Tyr Gln

705 710 715

20 AGC AAG CTG TGA TATTGGCTGG ATAGAGCCAA AGAAAATGTG ATTAGTAAGG
 1059 Ser Lys Leu *
 720

25 TAGTGTCTTT GGTCAGTTG GTGTGTTAAG GAACAAATAA TAATAATTAG CGACTATGAA
 1119

30 TAGTTATTGT TAAA
 1133

(2) INFORMATION FOR SEQ ID NO: 4:

35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 339 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: protein
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

45 Gln Arg Ser Leu Leu Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Ser
 1 5 10 15

50 Leu Ala Phe Leu Phe Tyr Tyr Ile Ala Thr Ser Tyr Phe His Leu Leu
 20 25 30

55 Pro His Pro Leu Ser Tyr Leu Ala Trp Ser Ile Tyr Trp Ala Leu Gln
 35 40 45

Gly Cys Ile Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His

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	50	55	60	
5	His Ala Phe Ser Asp Tyr Gln Trp Val Asp Asp Met Val Gly Leu Thr			
	65	70	75	80
	Leu His Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser His			
	85	90	95	
10	Cys Arg His His Ser Asn Thr Gly Ser Leu Asp Arg Asp Glu Val Phe			
	100	105	110	
15	Val Pro Lys Pro Lys Ser Lys Met Pro Trp Phe Ser Lys Tyr Phe Asn			
	115	120	125	
	Asn Pro Pro Gly Arg Val Leu Thr Leu Leu Ile Thr Leu Thr Leu Gly			
	130	135	140	
20	Trp Pro Leu Tyr Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp Arg			
	145	150	155	160
25	Phe Ala Cys His Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asn Arg Glu			
	165	170	175	
	Arg Cys Gln Ile Phe Val Ser Asp Ala Gly Val Phe Ala Thr Thr Tyr			
	180	185	190	
30	Val Leu Tyr Tyr Ala Ala Met Ser Lys Gly Leu Ala Trp Leu Val Phe			
	195	200	205	
35	Ile Tyr Gly Met Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile			
	210	215	220	
	Thr Tyr Leu Gln His Thr His Pro Ala Leu Pro His Tyr Asp Ser Ser			
	225	230	235	240
40	Glu Trp Asp Trp Leu Arg Gly Ala Leu Ala Thr Ala Asp Arg Asp Tyr			
	245	250	255	
45	Gly Met Leu Asn Lys Val Phe His Asn Ile Ile Asp Thr His Val Ala			
	260	265	270	
	His His Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr			
	275	280	285	
50	Lys Ala Ile Lys Ser Ile Leu Gly Lys Tyr Tyr Gln Phe Asp Gly Thr			
	290	295	300	

Pro Val Tyr Lys Ala Val Trp Arg Glu Ala Lys Glu Cys Leu Tyr Val
 305 310 315 320

5 Glu Ser Asp Glu Gly Ala Pro Asn Lys Gly Val Phe Trp Tyr Gln Ser
 325 330 335

10 Lys Leu *

15 **Claims**

1. A fragment of DNA from hazel (*Corylus avellana L.*) comprising the nucleotide sequence shown in Figure 2.
2. A DNA fragment comprising the nucleotide sequence shown in Figure 2 from base 222 to base 1367, which codes for the hazel $\Delta 12$ desaturase enzyme of the endoplasmic reticulum or for a homologous sequence which can code for the same amino-acid sequence.
3. A nucleotide sequence coding for a protein or peptide having an amino-acid homology greater than or equal to 80% and preferably greater than 90% with the hazel $\Delta 12$ desaturase enzyme of the endoplasmic reticulum of Claim 2 and having the function of the said enzyme.
4. A recombinant DNA sequence comprising a DNA sequence according to Claims 1, 2 and 3, or a portion of such a sequence, together with sequences regulating expression.
- 30 5. A recombinant DNA molecule comprising a cloning vector in which a DNA sequence according to any one of Claims 1, 2, 3 and 4 is inserted.
6. A DNA molecule according to Claim 5, in which the cloning vector is a plasmid or a phage.
- 35 7. A DNA molecule according to Claim 4 or Claim 5 having the restriction map shown in Figure 1.
8. A host organism including a recombinant DNA molecule according to any one of Claims 3 to 6.
9. A host organism according to Claim 8, selected from a vegetable cell, an animal cell, and a micro-organism.
- 40 10. A genetically modified organism capable of expressing the FAD2-N gene, having the amino-acid sequence shown in Figure 2 from bp 222 to bp 1367, portions of this gene, or this gene conjugated with other molecules and containing sequences which can inactivate endogenous genes.
11. A hazel $\Delta 12$ desaturase enzyme of the endoplasmic reticulum having the amino-acid sequence shown in Figure 2 in substantially pure form.
- 45 12. A fusion polypeptide comprising the amino-acid sequence of the enzyme of Claim 11, in which the amino-acids additively connected thereto do not interfere with the desaturase activity or can easily be eliminated.
- 50 13. The use of the FAD2-N gene coding for the hazel $\Delta 12$ desaturase enzyme of the endoplasmic reticulum or of portions thereof for the isolation of enzymes having the function of hazel desaturase or of the desaturase of another species.
- 55 14. The use of the nucleotide sequences of the FAD2-N gene shown in Figure 2 for the construction of expression systems which can alter the fatty-acid content in hazel.

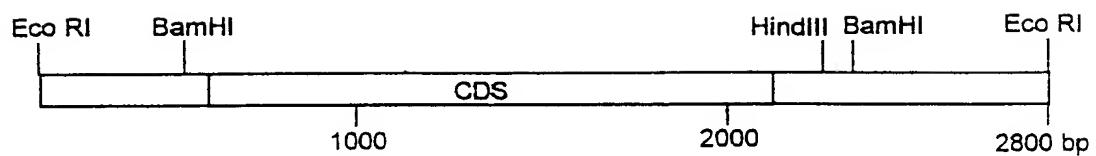


Fig. 1 - Restriction map of the genomic clone "N2". CDS: coding region; bp: base pair.

Fig. 2 - Nucleotide sequence of the gene FAD2-N corresponding to an internal fragment of the genomic clone "N2". Aminoacid residues of the coding region are also reported.

CCTCATAAAAAAAGTAAGCTCATTACCTCAAGTAGGGTTCTTATGACAAATGAGTCCC 60
GGAGTATTTTCATTGAGTAAATGGAGTTCATCCCAAAGGAATACTGTTACTCAGGG

GCAATCCTTTCTATGAGGTGCTATAATTGCAAATGTCCAAATCATAGGGATATGGATCC 120
CGTTAGGAAAAGATACTCCACGATATTAACGTTACAGGTTAGTATCCCTATACCTAGG

AAATACTATTAATATTATGTAGTGTGTCCCCCTCAAATTTACTCTCACACCT 180
TTTATGATAATTATAATACATCACACAAAAAAAGGGAGTTAAATGAGAGTGTGGA

AAGTTGATTTCTCCAGCATTGGACATAGCCTCTGTAGACAAATGGGAGCTAGAAGCCGAA 240
TTCAACTAAAAGAGGTCGTAACCTGTATCGGAGACATCTGTTACCCCTCGATCTCGGCTT

Met Gly Ala Arg Ser Arg

TGCCCTGCTACCAACAAGCTAAAGAGCAAAAAACACCCATCCAGCGAGCACCACACAA 300
ACGGACGATGGTTGTTGGATTTCGTTGGGTAGGTGCGTCGTGGTGTGTT

Met Pro Ala Thr Asn Lys Pro Lys Glu Gln Lys Thr Pro Ile Gln Arg Ala Pro His Thr

AACCCCCATTCACTCTTAGCCAACCTCAAGAAAGCCGTCCCACCCAAATTGTTCCAACGCT 360
TTGGGGTAAGTGAGAATCGGTTGAGTTCTTCGGCAGGGTGGGTTAACAAAGGTTGCGA

Lys Pro Pro Phe Thr Leu Ser Gln Leu Lys Ala Val Pro Pro Asn Cys Phe Gln Arg

CTCTCCTACGCTCGTTCTCATATGTTATGACCTCTCCTTAGCCTTCCCTTTCTACT 420
GAGAGGATGCCAGCAAGAGTATAACAACAAATACTGGAGAGGAATCGGAAGGAGAAGATGA

Ser Leu Leu Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Ser Leu Ala Phe Leu Phe Tyr

ATATTGCTACCTCTTACTTCCATCTCCTCCCTCACCCCCCTTCTACTTGGCATGGTCAA 480
TATAACGATGGAGAATGAAGGTAGAGGGAGGTGGGGAAAGGATGAACCGTACCAAGTT

Tyr Ile Ala Thr Ser Tyr Phe His Leu Leu Pro His Pro Leu Ser Tyr Leu Ala Trp Ser

TCTATTGGGCTCTCCAAGGCTGCATTCTCACCCGGCTTGGGTATCGCACATGAGTGCG 540
AGATAACCCGAGAGGTTCCGACGTAAGAGTGGCCGAAACCCAGTAGCGTGTACTCACGC

Ile Tyr Trp Ala Leu Gln Gln Cys Ile Leu Thr Gln Val Val Ile Ala His Glu Cys

GTCACCATGCCCTTACTGACTACCAATGGGTTGATGACATGGTTGGCCTAACCCCTTCAC 600
CAGTGGTACGGAAATCACTGATGGTTACCCAACTACTGTACCAACCGGATTGGGAAGTGA

Gly His His Ala Phe Ser Asp Tyr Gln Trp Val Asp Asp Met Val Gly Leu Thr Leu His

CTGCTTTAGTCCATACTTTATGGAAGATTAGCCACTGTCGCCACCACTCTAACAA 660
GACGAGAAAATCAAGGTATGAAAAGTACCTCTAATCGGTGACAGCGGTGGTGAGATTGT

Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser His Cys Arg His His Ser Asn

CCGGCTCCCTTGACCGAGATGAGGTGTTGCCCCAAGCCGAAATCCAAAATGCCATGGT 720
 GGCGAGGGAACTGGCTACTCCACAAACAGGGGTTCGGCTTAGGTTTACGGTACCA
 Thr Gly Ser Leu Asp Arg Asp Glu Val Phe Val Pro Lys Pro Lys Ser Lys Met Pro Trp

 TTTCTAAGTACTTCAACAAACCCACCAGGTAGGGTCCCTCACTCTTGATCACACTCACTC 780
 AAAGATTCACTGAAGTTGGTGGTCCATCCCAGGAGTGAGAAAACTAGTGAGTGAG
 Phe Ser Lys Tyr Phe Asn Asn Pro Pro Gly Arg Val Leu Thr Leu Leu Ile Thr Leu Thr

 TAGGCTGGCCCTTGTACTTAGCCTTGAATGTTCTGGCCGACCCATGATCGTTTGCTT 840
 ATCCGACCGGGAACATGAATCGGAACCTACAAAGACCGGCTGGGATACTAGCAAAACGAA
 Leu Gly Trp Pro Leu Tyr Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala

 GCCACTATGATCCCTATGGCCCCATTATTCCAATCGCGAAAGGTGTCAAATATTTGCT 900
 CGGTGATACTAGGGATACCGGGTAAATAAGGTTAGCGCTTCCACAGTTATAAACAGA
 Cys His Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asn Arg Glu Arg Cys Gln Ile Phe Val

 CGGATGCTGGTGTCTTGTACTACAACCTATGTGCTTACTACGCAGCAATGTCAAAGGGC 960
 GGCTACGACCACAGAAACGATGTTGAATACACGAAATGATGCGTCGTTACAGTTTCCCG
 Ser Asp Ala Gly Val Phe Ala Thr Thr Tyr Val Leu Tyr Tyr Ala Ala Met Ser Lys Glu

 TGGCATGGCTTGTATTCACTTATGGTATGCCATTGCTCATAGTGAATGGCTTCCCTTGAT 1020
 ACCGTACCGAACATAAGTAAATACCATACGGTAACGAGTATCACTACCGAACAGATA
 Leu Ala Trp Leu Val Phe Ile Tyr Glu Met Pro Leu Leu Ile Val Asn Gly Phe Leu Val

 TAATCACCTACTTGACGCACACTCACCCCTGCATTGCCGCACTATGACTCATCAGAATGGG 1080
 ATTAGTGGATGAACGTCGTGAGTGGGACGTAACGGCGTAACTGAGTAGTGTCTTACCC
 Leu Ile Thr Tyr Leu Gln His Thr His Pro Ala Leu Pro His Tyr Asp Ser Ser Glu Trp

 ATTGGCTTAGGGGGCATTGGCGACGGCGGATAGAGATTACGGAATGCTGAATAAGGTTT 1140
 TAACCGAATCCCCCGTAACCGCTGCCGCCTATCTCTAAATGCCCTACGACTTATTCCAAA
 Asp Trp Leu Arg Gly Ala Leu Ala Thr Ala Asp Arg Asp Tyr Glu Met Leu Asn Lys Val

 TCCACAATATCATAGACACCCATGGCTCACCATCTCTCTACCATGCCATTAC 1200
 AGGTGTTAGTATCTGTGGTACACCGAGTGGTAGAGAAGAGATGGTACGGAGTAATGG
 Phe His Asn Ile Ile Asp Thr His Val Ala His His Leu Phe Ser Thr Met Pro His Tyr

 ATGCAATGGAAGCCACCAAAGCAATCAAGTCATATTGGCAAATACATACCAAGTTGATG 1260
 TACGTTACCTTCGGTGGTTCGTTAGTTCACTAACCGTTATGATGGTCAAACATAC
 His Ala Met Glu Ala Thr Lys Ala Ile Lys Ser Ile Leu Gly Lys Tyr Tyr Gln Phe Asp

 GCACTCCAGTTACAAGGCAGTGTGGAGGGAGGGCTAAAGAGTGCCATTGTTGAGTCGG 1320
 CGTGAGGTCAAATGTTCCGTCACACCTCCCTCCGATTCACGGAAATACAACTCAGCC
 Gly Thr Pro Val Tyr Lys Ala Val Trp Arg Glu Ala Lys Glu Cys Leu Tyr Val Glu Ser

ACGAGGGGCCCTAACAAAGGTTTCTGGTATCAGAGCAAGCTGTGATATTGGCTGG 136C
TGCTCCCCGGGGATTGTTCCACAAAAGACCATAGTCTCGTTGACACTATAACCGACC

Asp Glu Gly Ala Pro Asn Lys Gly Val Phe Trp Tyr Gln Ser Lys Leu

ATAGAGCCAAAGAAAATGTGATTAGTAAGGTAGTGTCTTGCTCAGTTGGTGTGTTAAG 144C
TATCTCGGTTCTTACACTAATCATTCCATCACAGAAACCAAGTCAAACACACAATT

GAACAAATAATAATTAGCGACTATGAATAGTTATTGTTAAACAAAATTCACCCTTAT 150C
CTTGTATTATTATTAATCGCTGATACTTATCAATAACAATTGTTAAGTGGGAATA

GTTTAGCAGGAACCTTTCTGGCTACACTTTTCGTATGAAAAGC3CATATTTTTAAT 156C
CAAATCGTCCTGAAAAGACCGATGTGAAAAAAAGCATACTTTCGCGTATAAAAAAATT

TGTTATATTGTTGACATTACTCAAGCTTCAAAATTAAATATCACAGAAAATATCCAATG 162C
ACAATATAACAAACTGTAATGAGTTGAAAGTTTAATTATAGTGTCTTTATAGGTTAC

TCGAAGGTTTCATTGTAGGTTGAAAACTTTATATTGAGGTGG 1662
AGCTTCCAAAGTAACATCCAACCTTTGAAATATAACTCCACC

tccaaacgcctctccctacgcctcgttctcatatgttggttatgacccctcc 50
 tttagcccttcctcttctactataatgtacccctcttccatctccccc 100
 tcacccccccttctccctacttggcatggccatcttatttggcccttcccaaggct 150
 gcattctcaccggcgtttgggtccatcgccacatggatgcggccacccatggcc 200
 tttagtgacatccctccatgggttgcgtgacatggtttggcccttcccttc 250
 tgctcttttagttccatactttccatggacatggccatggcccttgcgtgcccc 300
 cctctaccaccaccggctcccttgcaccggatgtgggtgttttgcgtccccccgg 350
 acatcccccacatggccatggtttcttaagtactttccaccaccaccaccaggtag 400
 ggtccctccctcttttgcacccctcaatcttcggatggcccttgcgtacttag 450
 ccttgactgtttctggccggaccctatgatcgttttgtttgcgttgcgtatgtat 500
 ccctatggccccccatttttatccatcgccacccggatgcgttgcgtatgtat 550
 ggatgtgtgggtttgcacccctatgtgcctttacttcggccggccatgt 600
 ccccaagggttggccatggcttgcattttatgcgtatgcgttgcgtatgtat 650
 gtgtgtttccatgtatcaatccatcttcggatgcgttgcgttgcgtatgtat 700
 attgcccccctatgccttcatccggatggatgcgttgcgttgcgttgcgtatgtat 750
 cgccggccggatccggatgcgttgcgttgcgttgcgttgcgttgcgtatgtat 800
 atagacaccccatgtggatccacatctttatccatatgccttcatccccc 850
 tgcacttggacggccccccggatccatggatccatcttgcgttgcgtatgtat 900
 agtttgcgttggccatccacatgcgttgcgttgcgttgcgttgcgtatgtat 950
 tgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgtatgtat 1000
 gatgtatccatgtatgcgttgcgttgcgttgcgttgcgttgcgttgcgtatgtat 1050
 ttatgtatccatgtatgcgttgcgttgcgttgcgttgcgttgcgttgcgtatgtat 1100
 actaacttcacccatataccatccatgtatgtatgtatgtatgtatgtatgtat 1133

Fig. 3 - Nucleotide sequence of cDNA clone "I".

Fig 4 - Nucleotide sequence alignment of clones "I" (I.SEQ) and "N2" (N2.SEQ).

```

1  ----- I.SEQ
1  CCTCATAAAAAGTAAGCTCATTTACCTCAAGTAGGGTTT N2.SEQ

1  ----- I.SEQ
41 CCTTATGACAAATGAGTCCCAGCAATCCTTTCTATGAGGT N2.SEQ

1  ----- I.SEQ
81 GCTATAATTGCAAATGTCCAATCATAGGGATATGGATCC N2.SEQ

1  ----- I.SEQ
121 AAATACTATTAATATTATGTAGTGTGTGTGTGTGTCCC N2.SEQ

1  ----- I.SEQ
161 TCAAAATTACTCTCACACCTAAGTTGATTTCCTCCAGCAT N2.SEQ

1  ----- I.SEQ
201 TGGACATAGCCCTCTGTAGACAAATGGGAGCTAGAAGCCGAA N2.SEQ

1  ----- I.SEQ
241 TGCCTGCTACCAACAGCCTAAAGAGCAAAACACCCAT N2.SEQ

1  ----- I.SEQ
281 CCAGCGAGCACCCACACACAAACCCCCATTCACTCTAGC N2.SEQ

1  ----- TCCAACAGCT I.SEQ
321 CAACTCAAGAAAGCCGTCCCAACCCATTGTTTCCAAACGCT N2.SEQ

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361 CTCTCCCTACGGCTCGTTCTCATATGTTTATGACCTCTC N2.SEQ

50 CTTAGCCCTCCCTCTTCTACTATATTGCTACCTCTTACTTC I.SEQ
401 CTTAGCCCTCCCTCTTCTACTATATTGCTACCTCTTACTTC N2.SEQ

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561 TACCAATGGGTTGATGACATGGTTGGCCTAACCCCTTCACT N2.SEQ

250 CTGCTCTTTAGTCATACCTTTCATGGAAAGATTAGCCA I.SEQ
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330 GAGGTGTTGTCACCGAACGCGAAATCCAAACATGCCATGGT I.SEQ
681 GAGGTGTTGTCACCGAACGCGAAATCCAAACATGCCATGGT N2.SEQ

370 TTTCTAAGTACTTCACAAACCCACCAAGGTAGGGTCCCTCAC I.SEQ
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801 GCCTTGAAATGTTCTGGCCGACCCCTATGATCGTTTGCTT N2.SEQ

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921 A C A A C T T A T G T G C T T T A C T A C G C A G C A A T G T C A A A A G G G C N2 .SEQ

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1	M G A G G R M P V P T S S K K S E T D T T K R V P C E K P P F S V G D L K K A I	L26296.PRO
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41	P P H C F Q R S V L R S F S Y V V Y D L T I A F C L Y Y V A T H Y F H L L P G P	L43921.PRO
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80	L S Y L A W S I Y W A L Q G C I L T G V W V I A H E C G H H A F S D Y Q W V D D	N2.PRO
81	L S F R G M A I Y W A V Q G C I L T G V W V I A H E C G H H A F S D Y Q I L L D D	L43921.PRO
81	L S Y L A W P L Y W A C Q G C V L T G I W V I A H E C G H H A F S D Y Q W L D D	L26296.PRO
120	M V G L T L H S A L L L V P Y F S W K I S H C R H H S N T G S L D R D E V F V P K	N2.PRO
121	I V G L T L H S A L L L V P Y F S W K Y S H R R H S N T G S L E R D E V F V P K	L43921.PRO
121	T V G L I F H S F L L V P Y F S W K Y S H R R H S N T G S L E R D E V F V P K	L26296.PRO
160	P K S K M P W F S K Y F I N N P P G R V L T L I T L L G W P L Y L A L N V S G	N2.PRO
161	Q K S C I K W Y S K Y L N N P P G R V L T L A V T L L G W P L Y L A L N V S G	L43921.PRO
161	Q K S A I K W Y G K Y L N N P L G R I M M L T V Q F V L G W P L Y L A F N V S G	L26296.PRO
200	R P Y D R F A C H Y D P Y G P I Y S N R E R C Q I F V S D A G V F A T T Y V L Y	N2.PRO
201	R P Y D R F A C H Y D P Y G P I Y S D R E R L Q I Y I S D A G V L A V V Y G L F	L43921.PRO
201	R P Y D G F A C H F F P N A P I Y N D R E R L Q I Y L S D A G I L A V C F G L Y	L26296.PRO
240	Y A A M S K G L A W L V F I Y G M P L L I V N G F L V L I T Y L Q H T H P A L P	N2.PRO
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361	E C I Y V E P D R E G D K K G V F W Y N N K L	L26296.PRO

Fig. 5 - Aminoacid sequence alignment of $\Delta 12$ desaturase from hazelnut (N2.PRO), *Arabidopsis* (L26296.PRO) and soybean (L43921.PRO). Homologous residues are boxed.

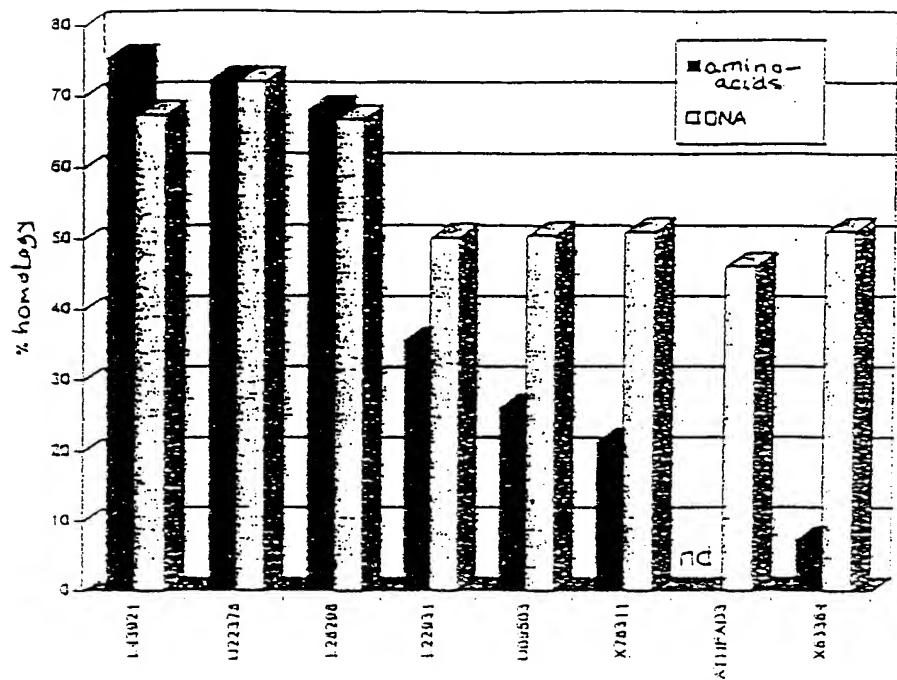


Fig. 6 - Homology between hazel $\Delta 12$ desaturase and other desaturases

- I43921: $\Delta 12$ desaturase of the endoplasmic reticulum of soya
- U22378: $\Delta 12$ hydroxylase of ricin
- L26296: $\Delta 12$ desaturase of the endoplasmic reticulum of *Arabidopsis thaliana*
- L22931: $\Delta 15$ plastid desaturase of *Arabidopsis thaliana*
- U09503: $\Delta 12$ plastid desaturase of *Arabidopsis thaliana*
- K78311: $\Delta 12$ plastid desaturase of spinach
- ATHFAD3: $\Delta 15$ desaturase of the endoplasmic reticulum of *Arabidopsis thaliana*
- X60364: $\Delta 9$ plastid desaturase of rape

Note: nc: not determined since the amino-acid sequence is not known.

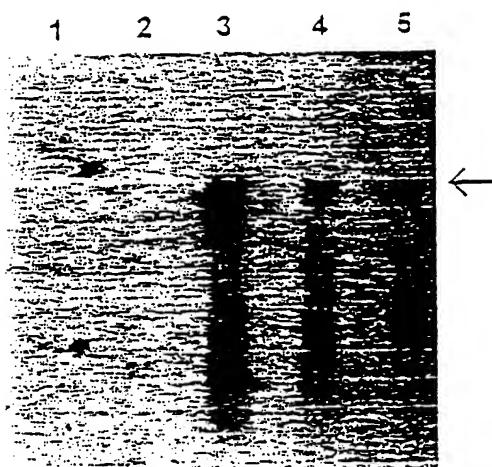


Fig. 7 - Northern blot of RNA of Montebello leaves (line 1), Nocchione leaves (line 2), Montebello kernels (line 3), Nocchione kernels (line 4), and San Giovanni kernels (line 5). The RNA was hybridized with the I clone of cDNA.



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 10 3098

DOCUMENTS CONSIDERED TO BE RELEVANT			Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)						
Category	Citation of document with indication, where appropriate, of relevant passages									
X	WO 94 11516 A (DU PONT ;LIGHTNER JONATHAN EDWARD (US); OKULEY JOHN JOSEPH (US)) 26 May 1994 examples 1,6,7 ---		10,13	C12N15/53 C12N15/82 C12N9/02 C12N5/10 C12Q1/68 //A01H5/00						
A,D	THE PLANT CELL, vol. 6, January 1994, pages 147-158, XP002034147 OKULEY, J., ET AL. : "ARABIDOPSIS FAD2 GENE ENCODES THE ENZYME THAT IS ESSENTIAL FOR POLYSATURATED LIPID SYNTHESIS" * page 155, column 2, line 28 * ---		1-14							
A	WO 95 22598 A (DU PONT ;LETO KENNETH JOSEPH (US); ULRICH JAMES FRANCIS (US)) 24 August 1995 * page 10, line 1 * -----		1-23							
				TECHNICAL FIELDS SEARCHED (Int.Cl.6)						
				C12N						
<p>The present search report has been drawn up for all claims</p> <table border="1"> <tr> <td>Place of search</td> <td>Date of completion of the search</td> <td>Examiner</td> </tr> <tr> <td>THE HAGUE</td> <td>3 July 1997</td> <td>Holtorf, S</td> </tr> </table>					Place of search	Date of completion of the search	Examiner	THE HAGUE	3 July 1997	Holtorf, S
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THE HAGUE	3 July 1997	Holtorf, S								
CATEGORY OF CITED DOCUMENTS		<p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>								
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document										